# Combined genetic and epigenetic alterations of the TERT promoter affect clinical and biological behaviour of bladder cancer

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Abstract: In urothelial bladder cancer (UBC), risk stratification remains an important unmet need. Limitless self-renewal, governed by TERT expression and telomerase activation, is crucial for cancer progression. Thus, telomerase activation through the interplay of mutations (TERTp ) and epigenetic alterations in the TERT promoter may provide further insight into UBC behavior. Here, we investigated the combined effect of TERTp and the TERT Hypermethylated Oncological Region (THOR) status on telomerase activation and patient outcome in a UBC international cohort (n=237). We verified that TERTp were frequent (76.8%) and present in all stages and grades of UBC. Hypermethylation of THOR was associated with higher TERT expression and higher-risk disease in non-muscle invasive bladder cancers (NMIBC). TERTp alone predicted disease recurrence (HR: 3.18, 95%CI 1.84 to 5.51, p<0.0001) but not progression in NMIBC. Combined THOR /TERTp increased the risk of disease recurrence (HR 5.12, p<0.0001) and progression (HR 3.92, p=0.025). Increased THOR hypermethylation doubled the risk of stage progression of both TERTp and TERTp NMIBC. These results highlight that both mechanisms are common and coexist in bladder cancer and while TERTp is an early event in bladder carcinogenesis THOR hypermethylation is a dynamic process that contributes to disease progression. While the absence of alterations comprises an extremely indolent phenotype, the combined genetic and epigenetic alterations of TERT bring additional prognostic value in NMIBC and provide a novel insight into telomere biology in cancer. 

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# Combined genetic and epigenetic alterations of the *TERT* promoter affect clinical and biological behaviour of bladder cancer

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**Key Words**: Urothelial bladder cancer, telomerase, *TERT* promoter methylation, *TERT* promoter mutations, recurrence, progression

**Abbreviations:**

TERT – telomerase reverse transcriptase
TERTp$^{Mut}$ – TERT promoter mutations
THOR – TERT hypermethylated oncological region
THOR$^{high}$ - THOR hypermethylated
THOR$^{low}$ – THOR hypomethylated (or non-methylated)
Wild – Wild type
Mut – mutatnt
UBC – urothelial bladder cancer
NMIBC – non-muscle invasive bladder cancer
MIBC – muscle invasive bladder cancer
LG – low-grade tumors
HG – high-grade tumors

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**Novelty & Impact Statement**

Telomerase (*TERT*) is ubiquitously activated in cancer through complex relatively unknown mechanisms. In the *TERT* dependent bladder cancer, we uncover that hypermetylation of the *TERT* promoter (THOR) is a dynamic and progressive process during carcinogenesis and when combined with early *TERT* promoter mutations THOR affects disease progression and patient outcome. The collaborative effects of genetic and epigenetic alterations on TERT and tumor recurrence provide new insights into telomere biology and may be applicable to other tumor types.

Telomerase reverse transcriptase (*TERT*) activation is central to cancer cell immortalization. It acts, however, through relatively unknown mechanisms. In urothelial bladder cancer (UBC) in particular, *TERT* activation can occur in the presence or absence of mutation, raising questions about alternative activation mechanisms. This study shows that hypermethylation of the *TERT* promoter (THOR) plays a key part in UBC, being a dynamic and progressive process, with hypermethylation levels increasing with bladder cancer severity. Moreover, both hypermethylation and *TERT* promoter mutation contributed to increased telomerase expression. The findings provide insight into telomere biology in UBC and may be applicable to other tumors.

**Abstract**

In urothelial bladder cancer (UBC), risk stratification remains an important unmet need. Limitless self-renewal, governed by *TERT* expression and telomerase activation, is crucial for cancer progression. Thus, telomerase activation through the interplay of mutations (*TERT*p$^{Mut}$) and epigenetic alterations in the *TERT* promoter may provide further insight into UBC behavior. Here, we investigated the combined effect of *TERT*p$^{Mut}$ and the *TERT* Hypermethylated Oncological Region (THOR) status on telomerase activation and patient outcome in a UBC international cohort (n=237). We verified that *TERT*p$^{Mut}$ were frequent (76.8%) and present in all stages and grades of UBC. Hypermethylation of THOR was associated with higher *TERT* expression and higher-risk disease in non-muscle invasive bladder cancers (NMIBC). *TERT*p$^{Mut}$ alone predicted disease recurrence (HR: 3.18, 95%CI 1.84 to 5.51, p<0.0001) but not progression in NMIBC. Combined THOR$^{high}$/*TERT*p$^{Mut}$ increased the risk of disease recurrence (HR 5.12, p<0.0001) and progression (HR 3.92, p=0.025). Increased THOR hypermethylation doubled the risk of stage progression of both *TERT*p$^{wt}$ and *TERT*p$^{Mut}$ NMIBC. These results highlight that both mechanisms are common and coexist in bladder cancer **and while** *TERT*p$^{Mut}$ is an early event in

bladder carcinogenesis THOR hypermethylation is a dynamic process **that contributes** to disease progression. While the absence of alterations comprises an extremely indolent phenotype, the combined genetic and epigenetic alterations of *TERT* bring additional prognostic value in NMIBC and provide a novel insight into telomere biology in cancer.

**Introduction**

Urothelial bladder cancer (UBC) poses significant burden as it is responsible for 123,051 deaths annually and at any given time there are more than 500,000 UBC patients in the USA alone (1, 2). UBC is a remarkably heterogeneous disease, including non-muscle invasive (NMIBC) and muscle invasive disease (MIBC). Pathological tumor stage and grade drive prognostic predictions and ultimately, therapy recommendations. However, pathology alone is often insufficient to predict individual outcomes. Grade and stage are crude measures and many patients are over- or undertreated as a result. Unlike other cancer types, few molecular markers currently guide UBC management (3, 4).

At diagnosis the majority of UBC (75%) are NMIBC (Ta, T1), mostly low grade (LG). LG tumors are rarely lethal but recur locally with variable and unpredictable rates (5, 6). Muscle-invasive bladder cancers (MIBC – T2, T3, T4), on the other hand, almost universally high-grade (HG), can be lethal and associated with worse clinical outcomes (5, 6). A subset of NMIBC are HG and destined to progress to life-threatening MIBC. There is an unmet need to

improve the prediction of those patients with NMIBC at risk of progression to MIBC. The stakes are high as NMIBC can be managed with conservative therapy whereas MIBC requires either the removal of the bladder (cystectomy) or chemo-radiation (7).

Telomerase, the enzyme complex responsible for maintaining telomere length and genome integrity is responsible for cellular immortalization (a hallmark of cancer) (8-11). Telomerase activity is upregulated in 85-90% of all cancers (12, 13). Mutations in the promoter of the catalytic subunit of the enzyme, termed telomerase reverse transcriptase (*TERT*) are frequently observed in several cancers and drive telomere maintenance (14-16). In UBC, *TERT* promoter mutations (C to T transitions at chr5:1,295,228 and chr5:1,295,250) are more common than any other genetic alterations and can lead to increased *TERT* expression and telomerase activity (17-20). Importantly, *TERT* promoter mutations are associated with worse clinical outcome in most studies further highlighting the role of telomerase activation in tumor progression and recurrence (18-27). Noteworthy, not all *TERTp*$^{Mut}$ tumors display telomerase activation and non-*TERTp*$^{Mut}$ UBC may express *TERT* suggesting that the presence of additional mechanisms are necessary for telomerase activation in UBC (28).

Our group and others have identified a parallel epigenetic control of telomerase activation in cancer. Specifically, a region located upstream of the core mutation area, within the *TERT* promoter, termed THOR (***T****ERT* **H**ypermethylated **O**ncological **R**egion) is hypermethylated in many TERT-expressing cancers, is associated with telomerase activation and predicts

clinical outcomes in multiple tumor types (14, 29, 30). We therefore hypothesized that dual mechanisms activate telomerase in UBC. Here, we studied the interaction and contribution of both genetic (*TERTp*$^{Mut}$) and epigenetic (THOR) *TERT* promoter alterations to telomerase activation and prognosis in UBC, using a multi-institutional cohort.

## Material and Methods

### Open Access Data

The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov) database for UBC was analyzed. A single, probe located within THOR (cg11625005) was used for methylation analysis (Illumina Infinium 450k array). *TERT* expression data were evaluated from the gene expression dataset (polyA+IlluminaHiSeq) (details in Appendix).

### Patients

Patients' selection and pathological characteristics are presented in Supplementary Table S1. All patients underwent surgery (either transurethral bladder resection or radical cystectomy) and followed for a median period of 107.4 months (IQR: 32.4–266.8 months). 331 bladder tissue samples, from 331 patients (237 UBC and 94 normal urothelium) were collected and analyzed for THOR methylation, *TERT* promoter mutations and a subgroup for h*TERT* expression. Survival data was collected for the 237 patients with

UBC. On 10 of these patients, an additional tumor at recurrence/progression was analyzed.

**Molecular analysis of the *TERT* promoter**

Sanger Sequencing was used to determined *TERT* promoter mutation status. Samples were considered mutant (*TERT*p$^{Mut}$) if any of the mutations (1,295,228 G>A or 1,295,250 G>A) were present (Supplementary Table S2). Quantitative sodium bisulfite pyrosequencing was performed for THOR as previously described (30). *TERT* expression was performed with the QX200 Droplet Digital PCR system (see Appendix for details).

**Statistical analysis**

SAS version 9.4 was used for statistical analyses. THOR was initially evaluated as a continuous value to determine its association with normal and malignant urothelial bladder tissue and to further interrogate its association with stage, grade and high and low risk disease. For the prognostic model we dichotomized into high- and low-THOR-methylation groups by receiver operating characteristic (ROC) analysis. Clinical outcomes for the *TERT* promoter mutations and THOR methylation were determined by Kaplan-Meier Survival curves. Cox Proportional Hazards (CPH) models were used to assess univariate and multivariate significance (details Appendix).

## Results

### *TERT* promoter mutations are early and frequent events in UBC

*TERT* promoter mutations (*TERT*p$^{Mut}$) were highly prevalent (76.8%, n=182). The predominant alteration was g.1,295,228 C>T which accounted for 90.1% of all mutations. No mutations were found in normal urothelium (Table 1).

*TERT*p$^{Mut}$ were identified in all stages and grades of UBC [detected in 69.3% of NMIBC (n=135/199) and 73.4% of low-grade lesions]. *TERT*p$^{Mut}$ were identified in all metastatic (tumor positive) pelvic lymph nodes as well as in their corresponding primary tumor. Interestingly, *TERT*p$^{Mut}$ were also identified in some tumor negative lymph nodes (Supplementary Table S3).

Consistent with previous studies, some of these *TERT*p$^{Mut}$ UBC did not exhibit high *TERT* expression even when compared to UBC with a wild type promoter (*TERT*p$^{Wt}$) (Supplementary Fig.S1A, S1C) (18).

These data support an early oncogenic role of *TERT*p$^{Mut}$ and the possibility that other mechanisms may also upregulate *TERT* expression in UBC.

**THOR hypermethylation is a dynamic process in UBC tumorigenesis**

In order to evaluate the extent of THOR methylation and its effect on *TERT* expression in UBC we analyzed a representative CG site within THOR (cg11625005) in a cohort of MIBC from the TCGA (n=433). When compared to normal urothelium, MIBC had significantly higher methylation at THOR and higher *TERT* mRNA levels (Supplementary Fig.S2A, $p<0.0001$; Supplementary Fig.S2B, $p<0.0001$). THOR hypermethylated (THOR$^{high}$) was associated with considerably higher *TERT* mRNA levels in malignant tissue (Supplementary Fig.S2C, $p<0.0001$) further supporting the role of *TERT* promoter methylation in *TERT* transcriptional activation.

To directly test this observation we assayed multiple representative CG sites from THOR in normal and UBC samples from our multi-institutional cohort. THOR was hypermethylated (THOR$^{high}$) in 127 UBC (53.6%, Table 1) and significantly hypermethylated in tumors compared to benign histology in non-matched samples (Figure 1A, $p<0.0001$). Additionally, paired samples from the same surgical specimen revealed that THOR methylation is 2 times higher in the tumor region than the corresponding normal urothelium (Figure 1B).

Since UBC are stratified by invasiveness (T stage) and cellular morphology (grade) as predictors of tumor progression, we also tested THOR ability to distinguish stages and grades. THOR methylation was significantly higher in tumor tissue, even when comparing superficial lesions (Ta) with normal tissue

(Figure 1C, $p<0.0001$). THOR methylation levels are slightly higher in T1 disease than in Ta (Figure 1C, $p=0.049$) but do not reach the same significant difference verified between normal urothelium and Ta disease. Similarly, THOR methylation demonstrated a progressive pattern from normal urothelium to low-grade tumors and maintaining the same trend in high-grade tumors (Figure 1D). Clinically, THOR exhibited higher hypermethylation in high-risk (T1 and HG) when compared to low risk tumors (Supplementary Fig.S3, $p=0.034$)(31).

To further explore the changes in THOR over time, we assessed THOR methylation in UBC harvested from consecutive surgeries (at both the time of the initial diagnosis and the first recurrence or progression). A significant increase in THOR methylation levels was observed in tumors with stage progression ($p=0.018$, mean increase fold of 1.76), but not in non-progressive ones ($p=0.88$) suggesting that THOR is hypermethylated in tumors harboring *TERT* promoter mutations (Supplementary Table S4).

Finally, analysis of *TERT* expression revealed that higher levels of THOR methylation are related to higher levels of expression (Supplementary Fig.S1B, $p=0.049$). The highest *TERT* expression is observed when both alterations (methylation and mutations) are present (Supplementary Fig.S1C).

Overall, these data suggest that THOR methylation increases progressively during the earlier stages of UBC and both *TERT* promoter mutations and hypermethylation contribute to increased telomerase expression.

### *Prognostic value of TERT* promoter alterations in NMIBC

As expected, in these selected cohorts, pathological grade was predictive of progression (n=199, $p$=0.02) (Supplementary Fig.S4).

As THOR methylation increases with disease stage while *TERT*p$^{Mut}$ is an early event, we assessed the value of *TERT* promoter alterations as markers of recurrence and disease progression in NMIBC.

Consistent with previous reports, patients harboring *TERT*p$^{Mut}$ had a significantly higher risk of recurrence (HR: 3.18, 95% CI 1.8-5.5; $p$<0.0001; Table 2) with significantly decreased median disease free survival (Log rank $p$<0.0001; Figure 2A)(17, 18, 25). However, *TERT*p$^{Mut}$ status did not reach statistical significance with respect to the risk of progression to invasive disease ($p$=0.052) (Figure 2B, Table 2)(17, 18).

As for *TERT*p$^{Mut}$, THOR$^{high}$ NMIBC recurred more frequently (HR: 1.5, 95% CI 1.02-2.20; $p$=0.03; Table 2), with decreased median disease-free survival (76 months vs. 31.7 months) (Log rank $p$=0.034; Figure 2C). Also, THOR$^{high}$ did not reach significance for the risk of progression (Log rank $p$=0.059; Figure 2D, Table 2) in NMIBC.

### Combined *TERT* promoter alterations predict disease progression in NMIBC

We then analyzed the combined prognostic impact of both *TERT* promoter alterations in NMIBC. We first evaluated the association of *TERT* promoter alterations and clinical outcomes according to grade and stage of NMIBC.

THOR$^{high}$/*TERT*$_p^{Mut}$ was the most common phenotype in NMIBC which recurred or progressed independently of stage (Supplementary Fig.S5 and S6). The absence of any alteration (THOR$^{low}$/*TERT*$_p^{Wt}$) was associated with a more indolent phenotype even in HG NMIBC (Supplementary Fig.S6).

Concomitant THOR hypermethylation and mutations (THOR$^{high}$/*TERT*$_p^{Mut}$) were associated with increased risk of disease recurrence (HR: 5.12; 95% CI 2.23-11.32, Table 2) with less than 30% disease free survival (DFS) at 10 years. In contrast, the absence of both alterations (THOR$^{low}$/*TERT*$_p^{Wt}$) was associated with 80% DFS at 10 years (Figure 3A, Log rank $p$<0.0001). The presence of either *TERT* promoter alterations (*TERT*p$^{Mut}$ or THOR$^{high}$) increased the risk of disease recurrence with a worse disease free survival (30.8% and 45% at 10 years, respectively; Figure 3A).

The presence of either *TERT* promoter alterations conferred a 4.53 fold increased risk of recurrence (95% CI 2.05-10.01; $p$=0.0002) but not for progression ($p$=0.14). Combined THOR$^{high}$/*TERT*$_p^{Mut}$ was a risk factor for both recurrence (HR: 5.4; CI 95% 2.42-12.04; $p$<0.0001) and progression (HR: 4.01; CI 95% 1.19-13.5; $p$=0.024;Table 2). Furthermore, THOR$^{low}$/*TERT*$_p^{Wt}$ NMIBC patients had a 91.4% progression free survival (PFS) at 10 years of follow-up, significantly better than THOR$^{high}$/*TERT*$_p^{Mut}$ (66.4% PFS; Log rank $p$=0.019; Figure 3B). When adjusting for stage (T1 *vs.* Ta), grade (HG vs LG), gender and age, THOR$^{high}$/*TERT*$_p^{Mut}$ also displays a trend towards significance (HR: 3.32, CI 95% 0.99-11.16; p=0.05) (Supplementary Table 5).

We then tested the effect of continuous increase in THOR methylation to enhance the predictive ability of disease progression in NMIBC, adjusting for *TERT* promoter mutations status. THOR levels affected disease progression in both *TERT*p$^{Mut}$ and *TERT*p$^{Wt}$ phenotypes. Increased THOR methylation from 10% (normal) to 50%, more than doubled the risk of disease progression independently of *TERT* promoter mutation status (Figure 4).

**Discussion**

This study is the first to assess concomitant *TERT* promoter alterations in UBC. Our results suggest a temporal and cooperative association between *TERT* promoter mutations and methylation, impacting telomere biology and clinical outcome.

*TERT* expression is upregulated in 85-90% of tumors via multiple molecular mechanisms including somatic mutations, *TERT* amplifications, *TERT* structural variants and epigenetic modifications through *TERT* promoter methylation (14). Our data reveal that 76.8% of UBC cancers harbor *TERT*p$^{Mut}$ and 53.6% have THOR$^{high}$, and 24.6% (n=49/199) of LG NMIBC acquired both alterations . The kinetics and interaction of these alterations in UBC, and the resulting effect on *TERT* expression are still unknown and important to decipher.

The observation that *TERT*p$^{Mut}$ are found ubiquitously across all stages and grades of UBC, even in low stage and grade lesions with low *TERT* expression, suggests that *TERT*p$^{Mut}$ is an early event necessary but insufficient by itself to drive disease progression. In fact other studies also verified that *TERT* promoter mutant UBC might express low mRNA levels (18). Similar data exist in other tumors where *TERT* promoter mutations are common. For example, in gliomas, *TERT*p$^{Mut}$ are detected in lower grade lesions where TERT expression and self-renewal are low (32,33).

*TERT*p$^{Mut}$ is necessary but not sufficient to maintain telomere length or telomerase upregulation (28). In *TERT*p$^{Mut}$ tumors additional alterations are likely required to upregulate telomerase and promote tumor progression.

We previously showed that THOR hypermethylation is a dynamic process during gliomagenesis and prostate cancer progression (29, 30). In UBC, *TERT*p$^{Mut}$ is an early event while THOR hypermethylation is associated with disease progression and increased *TERT* expression. This pattern has been observed in other cancer types (14, 29, 30).

A weakness of the WHO 2004/2016 pathological classification of NMIBC is that it gives almost no prognostic information in T1 patients as nearly all are classified as HG (34). In this study, *TERT* promoter alterations add significant value as prognostic biomarkers. For example, T1 THOR$^{low}$/*TERT*p$^{Wt}$ tumors, including HG lesions, carry a risk of progression of less than 10%, in sharp contrast with the 52% of progression for THOR$^{high}$/*TERT*p$^{Mut}$. If confirmed in prospective studies, this may help guide management when choosing conservative vs. aggressive therapy for these patients. Given the high recurrence rate in NMIBC, the identification of patients with very low potential for disease recurrence and progression might change the costly and invasive follow-up protocols in favor of individualized strategies.

In contrast, THOR$^{high}$/*TERT*p$^{Mut}$ significantly increased the chance of NMIBC recurrence and is a risk factor for disease progression across stages and grades. Furthermore, for the different *TERT* promoter mutation status, continuous THOR hypermethylation increases the risk of disease progression,

reinforcing the dynamic and crucial role of THOR methylation in bladder cancer tumorigenesis.Further supporting the role of THOR methylation, tumors exhibited higher THOR methylation at the time of stage progression.

Together, our findings support the hypothesis that *TERT*p$^{Mut}$ are early triggers in tumorigenesis which require cooperation with other events including THOR hypermethylation to ensure telomerase activation, immortality and disease progression.

The exact mechanism of telomerase activation by promoter hypermethylation is still under investigation. One possible explanation is that methylation leads to three-dimensional changes in local chromatin architecture resulting in increased transcription (35). Alternatively, methylation of this distal part of the promoter might also prevent the binding of transcriptional repressors, which in turn enables *TERT* expression (36). These mechanisms ought to be explored further as they may result in the development of novel targeted therapies. Demethylating agents have shown some encouraging results in cancers where THOR is hypermethylated, and may have a role in decreasing the risk of progression of NMIBC (37).

Our study has limitations due to its retrospective nature, patient selection, absence of centralized pathology review (although we chose specifically the HG vs LG 2004/16 WHO classification less prone to inter-observer variability) (34). Importantly, one should interpret the univariate and multivariate analysis with caution as Ta ant T1 tumors are treated differently. Furthermore, since large numbers of tumors are necessary for survival analysis in NMIBC, in our

multivariate analysis THOR$^{High}$/TERTp$^{Mut}$ only showed a trend towards significance as a risk factor for disease progression. Future studies with higher number of NMIBC tumors are needed to empower and statically validate our preliminary findings. Similarly, the limited numbers of patients with muscle invasive disease prevented a meaningful evaluation of this subgroup. However, since most of the prognostic role of *TERT* activation through THOR hypermethylation is observed during early stages of tumor development in multiple cancers, and NMIBC was the focus of the present study, our results suggest a similar process in UBC carcinogenesis

In summary, this study further supports the role of epigenetic control of the *TERT* promoter by THOR hypermethylation as a dynamic and progressive process in carcinogenesis, including UBC. The concomitant evaluation of *TERT* promoter mutation-methylation in NMIBC has identified a group with more indolent outcome, independently from grade or stage. Additional prospective studies should confirm that THOR$^{low}$/*TERT*p$^{Wt}$ tumors have a reduced risk of recurrence and progression to invasive disease. This should be explored in other tumor types known for harboring *TERT* promoter mutations and telomerase upregulation. A better understanding of the interplay between these two tumor-specific *TERT* activating mechanisms might improve clinical management in UBC and other *TERT*-dependent cancers.

## Additional Information

### Ethics approval and consent to participate

Research Ethics Board of each Institution involved approved this study.

### Conflict of Interest

The authors do not have any conflicts of interest**.**

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**Figure Legends.**

**Figure 1. THOR methylation in urothelial bladder cancer.**

**A)** THOR methylation status in normal urothelium and tumor tissue in the entire cohort (****, $p$<0.0001) **B)** The ratio of tumor/normal tissue in paired samples from the same patient (n=38). Note a mean of 2 fold increase in THOR methylation status in the malignant tissue. **C**) THOR methylation status in different tumor stages. **D)** THOR methylation status is within tumor grades, but low grade has significantly higher THOR methylation than normal urothelilum (****, $p$<0.0001). NS=non significant. Error bars represent median and Interquartile range (IQR).

**Figure 2. Survival estimates for patients with NMIBC stratified by *TERT* promoter alterations.**

**A)** Disease free and **B)** progression free survival for patients with NMIBC stratified by *TERT* promoter mutation status. **C)** Disease free and **D)** progression free survival for patients with NMIBC stratified by *TERT* promoter methylation status. $THOR^{low}$=THOR hypomethylated, $THOR^{high}$=THOR hypermethylated. $TERTp^{Wt}$=*TERT* promoter wild type (for the two studied mutations); $TERTp^{Mut}$ = *TERT* promoter mutant. Yellow =$TERTp^{Wt}$ and $THOR^{low}$; Blue= $TERTp^{Mut}$ and $THOR^{high}$

**Figure 3**. **Survival analysis of combined *TERT* promoter alterations in patients with NMIBC.** Kaplan-Meier analysis for **A)** disease free and **B)** progression free survival stratified by combined *TERT* promoter mutations and THOR methylation status in non-muscle invasive bladder cancer patients. $THOR^{low}$/*TERT*$p^{Wt}$ = blue; $THOR^{low}$/*TERT*$p^{Mut}$=light red;$THOR^{high}$/*TERT*$p^{Wt}$ =green; $THOR^{high}$/*TERT*$p^{Mut}$=dark red.

**Figure 4. Estimated probability of disease progression based on THOR methylation levels.** Risk of progression is estimated by any *TERT* promoter mutation status (wild type or mutant) as a result of increased THOR methylation. Red-*TERT* promoter mutant NMIBC, Black- *TERT*$p^{Wt}$ NMIBC.

**Table 1. Summary of *TERT* promoter methylation (THOR) and *TERT* promoter mutation status.**

| **THOR Methylation** | | | | |
|---|---|---|---|---|
| **$THOR^{low}$** | 110 | | 46.4% | |
| **$THOR^{high}$** | 127 | | 53.6% | |
| **Stage** | **$THOR^{low}$** | | **$THOR^{high}$** | |
| **Ta** | 45 | 43.7% | 58 | 56.3% |
| **T1** | 49 | 51% | 47 | 49% |
| **≥ T2** | 16 | 42.1% | 22 | 57.9% |
| **Total** | 110 | 46.4% | 127 | 53.6% |
| **Grade** | **$THOR^{low}$** | | **$THOR^{high}$** | |
| **Low Grade** | 76 | 56.3% | 59 | 43.5% |
| **High Grade** | 28 | 27.5% | 74 | 72.5% |
| **Total** | **104** | **43.9%** | **133** | **56.1%** |
| ***TERT* promoter mutations** | | | | |
| ***TERT*$p^{MutStatus}$** | | | | |
| ***TERT*$p^{Wt}$** | 55 | | 23.2% | |
| ***TERT*$p^{Mut}$** | 182 | | 76.8% | |
| ***TERT* promoter mutations (per mutation)** | | | | |
| **C228T** | 164 | | 90.1% | |
| **C250T** | 18 | | 9.9% | |
| **C228T/C250T** | 0 | | 0% | |
| **Total** | 182 | | 100% | |

| Stage | Wt (n/%) | | Mutant (n/%) | |
|---|---|---|---|---|
| **Ta** | 28 | 27.2% | 75 | 72.8% |
| **T1** | 23 | 24.0% | 73 | 76.0% |
| **≥ T2** | 4 | 10.5% | 34 | 89.5% |
| **Total** | **55** | **23.2%** | **182** | **76.8%** |
| **Grade** | **Wt (n/%)** | | **Mutant (n/%)** | |
| **Low Grade** | 36 | 26.7% | 99 | 73.4% |
| **High Grade** | 19 | 18.6% | 83 | 81.4% |
| **Total** | 55 | 23.2% | 182 | 76.8% |

$THOR^{low}$ =THOR hypomethylated; $THOR^{high}$ = THOR hypermethylated; $TERTp^{MutStatus}$ =*TERT* promoter mutation status; $TERTp^{Mut}$ = *TERT* promoter mutation; $TERTp^{Wt}$ =*TERT* promoter wild type.
Frequency of *TERT* promoter mutations (wild type and mutant) and THOR methylation (hypomethylated and hypermethylated) according to stage and grade disease.

## Table 2. Univariate Cox proportional hazards regression analysis of time for disease recurrence and disease progression in NMIBC (n=199).

| | Disease Recurrence | | | | Disease Progression | | | |
|---|---|---|---|---|---|---|---|---|
| | **HR** | **95% CI** | **Chi Sq** | ***P*** | **HR** | **95% CI** | **Chi Sq** | ***P*** |
| ***TERTp*$^{MutationStatus}$** | | | | | | | | |
| $TERTp^{Wt}$ (n=51) | Ref | | | | Ref | | | |
| $TERTp^{Mut}$ (n=148) | 3.18 | 1.8 to 5.5 | 17.16 | <0.0001 | 2.36 | 0.99 to 5.60 | 3.82 | 0.052 |
| **$THOR^{Methy}$** | | | | | | | | |
| $THOR^{low}$ (n=105) | Ref | | | | Ref | | | |
| $THOR^{high}$ (n=94) | 1.50 | 1.0 to 2.2 | 4.37 | 0.03 | 1.81 | 0.97 to 3.35 | 3.52 | 0.057 |
| **$THOR^{Methy}$/$TERTp^{MutStatus}$** | | | | | | | | |
| $THOR^{low}$ /$TERTp^{Wild}$ (n=35) | Ref | | | | Ref | | | |
| $THOR^{low}$ /$TERTp^{Mut}$ + $THOR^{high}$ /$TERTp^{Wild}$ (n=85) | 4.53 | 2.0 to 10 | 13.86 | 0.0002 | 2.64 | 0.77 to 9.1 | 2.38 | 0.123 |
| $THOR^{high}$ /$TERTp^{Mut}$ (n=79) | 5.12 | 2.3 to 11.3 | 16.33 | <.0001 | 3.92 | 1.2 to 13.0 | 4.97 | 0.025 |

HR=Hazard ratio; CI: confidence interval; Chi Sq= Chi Squared
$TERTp^{MutStatus}$ =*TERT* promoter mutation status (for the studied mutations); $TERTp^{Wt}$=*TERT* promoter wild type; $TERTp^{Mut}$ = *TERT* promoter mutant; $THOR^{Methy}$ = THOR methylation status; $THOR^{high}$=THOR hypermethylated; $THOR^{low}$=THOR hypomethylated